Review

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Biomarkers in Medicine

microRNAs as pharmacogenomic biomarkers for drug efficacy and drug safety assessment

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Much evidence has documented that microRNAs (miRNAs) play an important role in the modulation of interindividual variability in the production of drug metabolizing enzymes and transporters (DMETs) and nuclear receptors (NRs) through multidirectional interactions involving environmental stimuli/stressors, the expression of miRNA molecules and genetic polymorphisms. MiRNA expression has been reported to be affected by drugs and miRNAs themselves may affect drug metabolism and toxicity. In cancer research, miRNA biomarkers have been identified to mediate intrinsic and acquired resistance to cancer therapies. In drug safety assessment, miRNAs have been found associated with cardiotoxicity, hepatotoxicity and nephrotoxicity. This review article summarizes published studies to show that miRNAs can serve as early biomarkers for the evaluation of drug efficacy and drug safety.

Keywords: biomarker • cardiotoxicity • drug efficacy • drug metabolizing enzymes • drug safety • hepatotoxicity • microRNA • nephrotoxicity

MicroRNAs (miRNAs) are the family of small (usually 20–24 nucleotides long) noncoding RNAs comprising up to 2% of mammalian genomes. MiRNAs can regulate gene expression post-transcriptionally via their interactions with partially complementary sequences found in the 3'-untranslated regions (3'-UTRs) of mature mRNA transcripts. Currently, over 35,000 miRNAs found in 223 species are listed in the miRBase, of which, approximately 2500 miRNA have been found in humans (miRBase, Version 21). Each miRNA species is predicted to regulate a number of targets due to the relaxed target sequence complementarity involved, often with over a hundred putative targets per one miRNA, with some estimates reaching over 200 mRNA transcripts regulated by a single miRNA [1]. On the other hand, one gene may be regulated by multiple miRNAs, adding another layer of complexity into miRNA-gene regulatory networks.

MiRNAs may originate from a variety of RNA molecules that may be regulated and transcribed in different ways, but that are processed to form mature miRNA species via a conserved pathway. Intronic miRNAs are those that are generated from sequences found within introns of known genes, while intergenic miRNAs are produced from intergenic regions. The longer initial miRNA primary transcripts (pri-miRNA) are generated by RNA polymerases II and III directly from genomic DNA. After transcription, each pri-miRNA forms a stem-loop structure or hairpin that is further processed by the RNAse III enzyme Drosha to create a precursor miRNA (pre-miRNA). Exportin-5 then transports the pre-miRNAs from the nucleus to the cytoplasm. Here, they are processed by another RNAse III enzyme, Dicer and are bound by an Argonaute proteins (AGO1–AGO4 in humans) and incorporates into an RNA-induced silencing complex (RISC). Following unwinding, one miRNA strand is degraded and the remaining one becomes a mature miRNA. The detailed biogenesis of miRNAs can be found elsewhere in excellent reviews [2–4].

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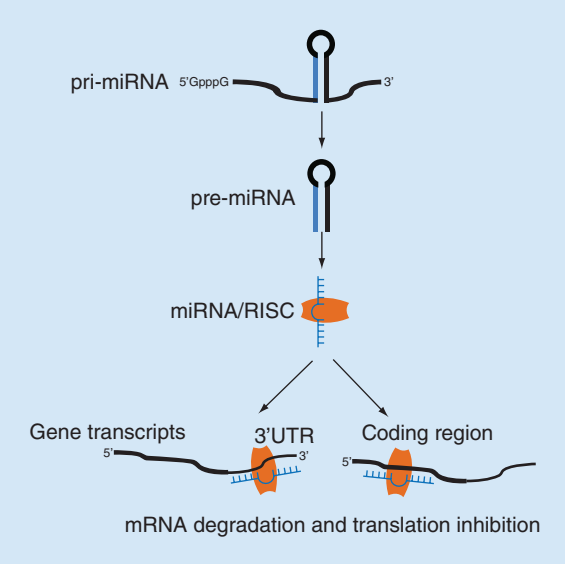

Figure 1. The process of miRNAs in gene modulation. The miRNA primary transcript (pri-miRNA) forms a stem-loop structure or hairpin that is processed by the RNase III enzyme (Drosha) to create a precursor miRNA (pre-miRNA), and then processed by another RNase III enzyme (Dicer) to create mature miRNA. Mature miRNA is bound by an Argonaute proteins (AGO1–AGO4 in humans) and incorporates into an RNA-induced silencing complex (RISC). The roles of miRNAs in gene silencing might include inducing mRNA degradation or preventing mRNA from being translated.
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MiRNAs usually regulate mRNA targets via imperfect base pairing to sequences found in the 3'-UTRs of their target mRNA transcripts. Hybridization efficiency for miRNA/target binding is determined by a seed-sequence – a stretch of six nucleotides covering nucleotides 2–7 within the miRNA 5'-end [5]. As shown in Figure 1, this results in post-transcriptional repression through mRNA destabilization (more frequently) or inhibition of translation (more rare); however, both mechanisms operate to decrease the expression of the target protein [4,6].

Because the vast majority of protein coding genes are predicted to be targeted by miRNAs [7], it has been proposed that all biological processes can be regulated by them [4]. Indeed, miRNAs have been shown to be involved in the control of a number of fundamental cellular processes, including differentiation, proliferation, apoptosis and signal transduction. Significant regulatory roles for miRNAs in the development of important diseases, such as cancer, as well as in a variety of cellular responses to hazardous or therapeutic agents are documented. Recently, studies have reported that exposure to various environmental toxicants and pharmaceuticals can lead to alterations in miRNA expression and subsequent alterations in expression of genetic information [8,9].

Several mechanisms may affect the expression of miRNAs and their targets in the cell. These include mutations in mature miRNAs or in their mRNA target sequences, along with deletions and epigenetic alterations (i.e., changes in DNA methylation) in miRNAs that can result in altered expression, which may impact the development of disease. The mechanistic bases for alterations in miRNA-dependent regulatory pathways require further education to provide a more robust understanding of gene regulation. However, the identification of alterations in miRNA expression in response to exogenous stressors may provide a valuable tool for the development of surrogate biomarkers of exposure and tissue/organism response to these stressors. MiRNAs have been broadly studied in physiological and pathological processes and their expression profiles have been used as biomarkers to be associated with diseases, such as various types of cancer [10,11], heart diseases [12], mental disorders [13], metabolomic disorders [14] and so forth. In addition, miRNAs have also been explored as potential therapeutics for disease treatments [15,16]. In this review, we will concentrate on miRNAs as potential biomarkers for the assessment of drug efficacy and safety.

Impact of miRNA on pharmacogenomics: interindividual variability of DMETs by miRNA modulation

Humans, exhibit a huge diversity in drug sensitivity, efficacy and toxicity [17–19]. Only 25–60% of patients show pharmacological responses to a specific medication [18] and more than 2 million adverse drug reaction cases are reported in the USA each year [20]. The reasons for interindividual variability in drug responses, or drug efficacy and side effects may be due to genetic variants (such as single nucleotide polymorphisms and copy number variations), environmental stimuli (such as dietary components), epigenetic modulation of gene expression by methylation or by noncoding RNA molecules, disease/health conditions of the individuals, or drug–drug interactions. In a given individual, these multifactorial components may influence drug absorption, metabolism and pharmacokinetics, to create interindividual variability in drug efficacy, safety and adverse drug reactions [17,18,21,22].

Establishing a more thorough understanding of the interindividual variability in the expression of drug-metabolizing enzymes and transporters (DMETs) is a critical step toward improving drug efficacy and minimizing adverse reactions. During the last several decades, studies have investigated the relationship between genetic polymorphisms and the expression

of DMETs [23–25]. Based on these findings, the US FDA has provided a database of genetic variants that may affect the treatment outcomes for certain drugs in the database. However, the involvement of genetic variation in gene expression does not explain fully the interindividual variability in drug responsiveness or expression of DMETs. The influence of epigenetic factors on gene expression, such as DNA methylation, post-translational histone modification and noncoding regulatory RNAs, may offer a new avenue for investigating the mechanisms underlying the interindividual variability in the expression of DMETs. In addition to genetic polymorphisms and the dynamics of DNA methylation and chromatin status, the modulation of protein translation by miRNAs exhibits a precisely controlled mechanism to fine-tune gene expression patterns in tissues/cells. Therefore, as epigenetic modulators, miRNAs play an important role in gene regulatory networks that are relevant to drug absorption, metabolism and disposition. Consequently, the differential expression of miRNA molecules among individuals could lead to a substantial change in individual phenotypes, resulting in a significant influence on quantitative traits, such as the sensitivity, efficacy and safety of therapeutic agents [26–29].

As shown in Figure 2, the central dogma of miRNA in modulation of drug efficacy and safety is a complex process that is a consequence of multidirectional interactions among environmental stimuli/stressors, the expression of miRNA molecules and the production of DMET and nuclear receptor (NR) proteins. First, the expression of miRNA molecules, NRs and DMETs is directly susceptible to environmental stimuli/stressors; second, the expression of NRs and DMETs could be mediated indirectly by environmental stimuli/stressors of miRNAs. Multiple studies have shown that xenobiotics influence the expression of NRs [30,31] and DMETs [32–34]. Similar to mRNA expression, the expression of miRNAs is tightly controlled by transcription factors [35] that are responsive to environmental challenges, including dietary constituents or metabolites derived from the diet, pollutants and other xenobiotics [9,36]. In addition, the expressions of miRNAs, NRs and DMETs are modulated by single nucleotide polymorphisms (SNPs), which lead to great interindividual variability in drug efficacy and safety.

Dietary factors, such as vitamins and micronutrients, are critical for the dynamic balance of epigenetic patterns that keep cells healthy. For example, vitamins might be important regulators in the expression of certain miRNAs. A study using rats revealed that 6 months of a vitamin E deficient diet resulted in significant reductions in the hepatic expression of miRNA-122a and miRNA-125b, although a detailed molecular mechanism for the regulation was not established [37]. A methyl donor-deficient diet was shown to suppress the expression of miR-16a, miR-34a, miR127 and miR-200b specifically in a rat hepatocarcinogenesis model, indicating that folate could regulate the expression of certain miRNA molecules in rat liver, which might be relevant to the development of hepatocarcinoma in humans [38]. Natural agents, such as folic acid, curcumin, isoflavone, indole-3-carbinol and resveratrol and other dietary constituents can change the expression level of miRNAs (such as miR-7, miR-20b, miR-21, miR-130a, miR-146a) in human cells, leading to a series of biological and pathological changes, including the inhibition/suppression of cancer cell growth, induction/reduction of apoptosis, promotion/inhibition of mesenchymal differentiation and enhanced or decreased efficacy of conventional therapeutics [39]. MiRNA expression is clearly affected by the administration of common drugs, such as dexamethasone, vinblastine and fluoxetine and enoxacin [29]. Likewise, other xenobiotic stressors, including chemical pollutants (such as heavy metals, water disinfection byproducts, air pollutants, endocrine-disrupting toxicants and cigarette smoke), can also affect the expression of miRNA molecules [9,28,36].

Transcriptional regulation of DMETs mediated by families of NRs is one of the major mechanisms controlling the expression of DMET genes. Fourteen NRs, such as CAR (NR1I3), PXR (NR1I2), aryl hydrocarbon receptor (AhR) and peroxisome proliferator activated receptor-γ (PPAR-γ, or NR1C3) are involved in the transcriptional regulation of DMETs [40,41], which contribute significantly to the interindividual variability of DMET expression. In addition to genetic polymorphisms and environmental exposure, which could have contributed to the variable expression of nuclear factors, miRNAs also can modulate of the expression of NRs. Evidence has accumulated to show the post-transcriptional regulatory relationships between *PXR*/miR-148a, *VDR*/miR-125a and miR27b, *PPARγ*/miR-27a and miR-27b, *RXRα*/miR-27, *HNF4a*/miR-34a and miR-24 and ERα/miR-206. In addition to these interactions between miRNAs and NRs, many other miRNAs that are involved in the regulation of NRs have been reported [9,42], for example, Takwi *et al.* [43] reported that miRNA-137 overexpression could facilitate the degradation of mRNA that targets constitutive androstane receptor (CAR) gene. Furthermore, a negative feedback circuit was found, by which miRNA-137 downregulates CAR expression and CAR downregulates the expression of miR-137. Through modulating the expression of ATP-binding cassette transporters, miR-137 is a key regulatory molecule accounting for the interindividual variability in response to doxorubicin for chemotherapy.

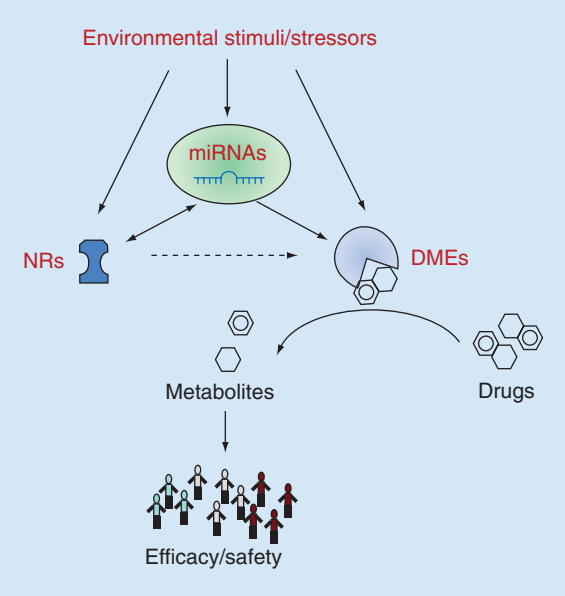

Figure 2. The central dogma of miRNA in modulation of drug efficacy and safety. First, the expression of miRNA molecules, NRs and DMETs are directly susceptible to environmental stimuli/stressors; second, the expression of NRs and DMETs could be mediated directly by miRNAs. In addition, the expression of miRNAs might be controlled by NRs.
DMETs: Drug metabolizing enzymes and transporters; NRs: Nuclear receptors.

During the last decade, there has been an increasing interest in the role of epigenetic factors, including the role of miRNAs, in the regulation of DMET expression. Studies have demonstrated that the epigenetic factors responsible for regulating the expression of DMETs are tissue-specific, sexually dimorphic, exhibit interindividual variability and are adaptive to xenobiotic exposure [44]. Since miRNAs are able to target a large number of mRNA encoded by the human genome and a given mRNA molecule could be targeted by multiple miRNAs, DMETs have been theoretically and experimentally suggested to be regulated by miRNAs, thus contributing to the interindividual variability in drug response and toxicity [9,45]. For example, *CYP1B1*/miR-27 [46], *SULT1A1*/miR-631 [47] and *CYP2C9*/miR-128 [48] have been demonstrated to have interactions that result in the suppression of DMET gene expression. Currently, multiple association/correlation studies have reported an inverse correlation between the expression of miRNAs and DMETs; however, fewer studies have used *in vitro* and/or *in vivo* approaches to investigate the molecular mechanisms by which miRNAs regulate the expression of their cognate DMETs. Yu [27], Rukov *et al.* [49], Kacevska *et al.* [50] and Kim *et al.* [51] summarized experimentally validated miRNAs that are involved in the modulation of the expression DMETs. As an update, we list newly published articles (from July 2013 to March 2015) in Table 1 reporting miRNA–DMET interactions based upon *in vitro* and/or *in vivo* evidence.

As important pharmacogenomic components, miRNAs manifest characteristics of interindividual variability in two respects: genetic polymorphisms altering binding sites for miRNAs in the mRNA transcripts they regulate and variability in miRNA expression among the population [49]. SNPs or insertions/deletions in the miRNA sequence, especially in the root region of a miRNA, could affect miRNA binding affinity to its cognate mRNA molecules, and *vice versa*. For example, the SNP rs4788068 located in the 3'-UTR of *SULT1A1* has different binding affinities with miR-631: the free energy of binding for the T allele is -31.4 kcal/mol and the free energy of binding for the C allele is -26.8 kcal/mol. Consequently, the mRNA transcripts for *SULT1A1* produced from the T allele are suppressed more efficiently by miR-631, and association analysis confirmed that the SNP in the 3'-UTR is significantly associated with SULT1A1 activity in Caucasians, suggesting miR-631 regulates *SULT1A1* expression in a genotype-specific manner [47]. Currently, a handful of databases, such as MirSNP [52], and PolymiRTS [53], provide information regarding the influence of genetic variants on the interaction between miRNAs and their cognate genes.

To explore the interindividual variability in the expression of miRNAs, a study selected 56 miRNAs that are relevant to DMET expression to perform miRNA expression analysis in 92 (40 males and 52 females) human liver samples [45]. The study demonstrated a large degree of interindividual variability in the expression of a set of miRNAs, including miR-539, miR-200c, miR-31, miR15a and miR-22, with a fold-change range from 1000- to 30,000 among the liver samples from different donors, in contrast to another group of four miRNAs that had a much smaller interindividual variability with a fold change less than tenfold. The expression of miR-31 in liver was correlated with sex, with a higher expression level in men than in women, and the expression of several other miRNAs was significantly correlated with age [45]. This miRNA profiling study with multiple samples from a human population suggested the interindividual variability in the miRNA expression might be partially due to confounding factors such as age, health status, lifestyle differences (dietary, smoking, alcohol and drug use) and other environmental exposures.

It is worthwhile to discuss how much a differentially expressed miRNA could contribute to the interindividual variability of DMET expression. We believe that genetic variation, chemical induction and epigenetic modulation (including miRNA modulation) con-

Table 1. Experiment validated microRNAs in regulation of drug metabolizing enzymes and transporters.

Gene function	Gene ID	Interacting MicroRNA	Study [Ref.]
NR	*CAR*	miR-137	Takwi [43]
NR	*RXRA*	miR-34a	Oda [215]
Phase I	*CYP2A6*	miR-126*	Nakano [216]
Phase I	*CYP2C9*	miR-128	Yu [48]
Phase I	*G6PD*	miR-206	Coda [217]
Phase I	*HMGCR*	miR-21	Sun [218]
Phase I	*POR, GSR*	miR-214	Dong [219]
Phase I	*TYMS*	mir-433	Gotanda [220]
Phase II	*ACSL4*	miR-205	Cui [221]
Phase II	*NAT1*	miR-1290	Endo [222]
Transporter	*ABCA1*	miR-33a/b	Ma [223]
Transporter	*ABCA1*	miR-302a	Meiler [224]
Transporter	*ABCA1*	miR-19b	Lv [225]
Transporter	*ABCA1*	miR-613	Zhao [226]
Transporter	*ABCA1*	miR-27a/b	Zhang [227]
Transporter	*ABCB9*	miR-31	Dong [87]
Transporter	*ABCC1*	miR133a, miR-326	Ma [228]
Transporter	*ABCC1*	miR-1291	Pan [229]
Transporter	*ABCC4*	miR124a, miR-506	Markova [230]
Transporter	*ABCG2*	miR-145	Shi [231]
Transporter	*ATP7A*	Mir-495	Song [232]
Transporter	*SLC19A2*	miR-155	Kim [233]
Transporter	*SLC22A18*	miR-137	Zhang [234]
Transporter	*SLC2A3*	miR-106a	Dai [235]
Transporter	*SLC7A11*	miR-27a	Drayton [236]

tribute to the differential expression of DMETs, which is a gene-by-gene phenomenon. In some cases, genetic factors control gene expression or enzyme activity. For example, *CYP2C9*2* is a decreased function allele owing to a genetic variant in the coding region that results in a significantly decreased enzyme activity [54]. Although miR-128 was found to suppress the expression of *CYP2C9*, the efficacy of miR-128 suppression only contributed less than 10% of the variability in the human population, which is much less than the variability introduced by the genetic variant [48]. In other cases, genetic regulation and epigenetic modulation coordinately regulate gene expression/enzyme activity. For example, SNPs in the coding region [55], promoter region [56] and copy number variants [57] have been found in the *SULT1A1* gene however, all these genetic variations could only partially account for the inter-individual variability observed for SULT1A1 enzyme activity. Recently, miRNA-631 was found to be able to suppress the expression of *SULT1A1*, but the effect of miRNA-631 in regulation of *SULT1A1* is also limited. In general, the modulation of DMETs by miRNAs is important; however, considering the greater influences associated with copy number variations, genetic variations, DNA methylation and other important epigenetic mechanisms, the power of miRNA-mediated gene regulation should not be overstated.

Roles of miRNA species in mediating intrinsic & acquired resistance to cancer therapeutics

The progressive elucidation of numerous mechanistic roles for miRNAs in the regulation of tumor induction, development, progression to metastasis, diversity of tumor phenotypes from common cellular origins, and, notably, the resistance of tumor cells to anticancer drugs is continually changing and refining the classical biological perspectives of carcinogenesis and cancer

therapy. Resistance to effective anticancer treatment modalities by tumor cells represents a well-known and vexing problem for oncologists [for recent reviews see 58–60]. Because rational strategies for diagnosing, avoiding and overcoming drug resistance are critically important for successful patient care, this topic continues to be the subject of intensive basic and applied biomedical research involving the newest and most innovative methods available.

Intrinsic or primary drug resistance may be present innately in some individuals due to inherited traits [61,62]. As an example, Starczynski *et al.* [63] showed that an inherited single-nucleotide polymorphism (SNP) located in the promoter region of the *BAX* tumor suppressor gene (-248G-->A) is associated with decreased expression of BAX, reduced overall survival time and an impaired response to chemotherapy in patients diagnosed with chronic lymphocytic leukemia. Mishra *et al.* [64] discovered that a 829C-->T SNP adjacent to an miR-24 binding site in the 3'-untranslated region (3'-UTR) of the dihydrofolate reductase (DHFR) mRNA transcript disrupts the miR-24-dependent destabilization of *DHFR* mRNA. Cells bearing this 829C-->T SNP exhibited increased DHFR expression and a fourfold increase in resistance to methotrexate. The allele frequency observed for the *DHFR* 829C-->T SNP indicates that it is moderately common among the Japanese population (829T allele frequency 0.142, n = 120) [65] and the frequency for the *BAX* -248G-->A SNP is also relatively common among a study population in Birmingham, UK (248A allele frequency 0.10, n = 338), although the study sizes were somewhat small for these two examples.

Typically, acquired or secondary resistance to anticancer treatments is observed more frequently than intrinsic resistance. Unlike the situation for intrinsic resistance, which is restricted to individuals bearing specific genotypes predisposing them with resistance to cancer therapies, acquired resistance may develop in almost any patient as a consequence of the various selective pressures placed upon tumor cells to survive and proliferate. In addition to obvious selective pressures resulting from applied medical therapies, tumor microenvironments may be anoxic, acidic, depleted of extrinsic growth factors and include elements of the active and innate immune systems that are hostile to cancerous cells. Ultimately, these selective forces must be overcome by tumor cells in order for the disease to progress to fulminant metastatic cancer.

The ability of tumors to escape selective pressures via acquired resistance is due, at least in part, to the presence of phenotypic variations exhibited by different subpopulations of cells within tumors. Tumors are often comprised of mixed populations of cancer cells harboring a variety of genetic differences [66]. Chromosomal instability is a well-known hallmark of cancer that is associated with random malfunctions of the mitotic spindle within some dividing cells [reviewed in 67]. Abnormal chromosomal segregation occurring with subpopulations of tumor cells which escapes spindle assembly checkpoints introduces aneuploidy and genetically heterogeneous cellular progeny within a single tumor. The classical rationale for observed variations in clinically relevant phenotypic properties expressed by subpopulations of tumor cells, such as differing metastatic potential or enhanced drug resistance, is that strategic survival and growth advantages available to mutant subpopulations of cells are favored. According to this model, selective pressures facilitate the clonal expansion of tumor cells bearing favorable mutations, creating a dynamically changing mixture of cells within tumors.

Here, we will consider epigenetic alterations, specifically those involving miRNA, that may also provide similar phenotypic advantages for tumor cells. Because alternations in miRNA levels reflect reversible alterations in gene expression that occur in response to environmental factors, such as inflammation or hypoxia, changes in miRNA levels have the potential to develop prior to the subsequent chromosomal changes that are generally considered irreversible. Appreciating the roles of miRNA species in mediating intrinsic and acquired resistance to cancer therapies offers opportunities in cancer research for identifying useful miRNA biomarkers that may be indicative of tumor phenotypes that are resistant to specific treatment regimens [58,68]. It also provides opportunities to identify miRNA species that could be targeted by therapeutic agents to mitigate resistance to anticancer therapies [69]. Moreover, miRNA species are also involved in resistance to other forms of cancer treatments besides chemotherapeutics; mechanisms involving miRNA species have also been shown to provide resistance to radiotherapy and immunotherapy anticancer strategies (for recent reviews, see [70,71]).

Cancer cell exosomes facilitate transfer of miRNA species & other factors involved in drug resistance to recipient cells

Cancer cells are known to interact with normal cells via various secreted factors to recruit other cell types, including cancer-associated fibroblasts, vascular endothelial cells or tumor-infiltrating leukocytes, each of which is important for tumor development and progression to metastatic cancer [72]. In addition to the secretion of well-known signaling molecules, such as cytokines, chemokines and growth factors, cancer cells also secrete small (20–200 nm) membrane-bound

vesicles known as exosomes containing miRNA, mRNA, proteins and sometimes DNA produced by the donor cell. Uptake by normal cells of the exosome cargo secreted by cancer cells can affect the behavior of recipient cells in various ways that provide benefits to the tumor [73,74]. Chen *et al.* [75] showed that the survival of doxorubicin- and docetaxel-sensitive MCF-7 breast cancer cells was promoted by treatment with exosomes obtained from drug-resistant MCF7 cells that contained miR-100, miR-222 and miR-30a. In a study performed by Wang *et al.* [76], exosomes secreted by bone marrow stem cells induced resistance to bortezomib in RPMI 8226 human multiple myeloma cells. Umezu *et al.* [77] showed that the K562 human chronic myelogenous leukemia (CML) cell line secreted exosomes containing the miR-17–92 cluster (miR-17, -18a, -19a, -20a, -19b and -92a) to stimulate angiogenesis. Cultured human umbilical vein endothelial cells (HUVEC) exposed to the exosomes secreted by K562 CML cells exhibited increased HUVEC migration and tube formation along with a significant reduction in integrin 5α protein, whose mRNA is a target for the miR-17–92 cluster. Although additional research is needed on this topic, the emerging viewpoint is that subpopulations of drug-resistant cancer cells are able to enhance the survival of other cells via exosomal delivery of regulatory miRNA species, intact mRNA and proteins. By extending an umbrella of protection that limits selective pressure by cancer treatments, a greater opportunity is provided for additional tumor cells to develop traits that are conducive to survival, growth and malignancy.

MicroRNAs contribute to cancer drug resistance via enhanced drug efflux

Enhanced drug efflux via the overexpression of proteins belonging to the ATP-binding cassette transporter (ABC) family is a commonly observed mechanism involved in drug resistance by cancer cells (Tables 2–3). Among the most well-known of ABC transporters is the multidrug resistance protein 1 (MDR-1/ABCB1/P-gp), which is involved in the efflux of a variety of xenobiotic compounds and important cancer drugs, including etoposide, doxorubicin, vinblastine, cisplatin and paclitaxel. Direct targeting of *MDR-1* mRNA transcripts by miRNA species generally leads to reduced expression and decreased drug resistance. Decreased MDR-1 expression and reduced drug resistance is associated with higher levels of miR-27a, miR-129–5p, miR-298, miR-331–5p and miR-451; these miRNAs function via sequence-specific binding sites present in the 3′-UTR of *MDR-1* mRNA transcripts to decrease the half-life of *MDR-1* mRNA [78–81]. The indirect targeting of other factors by miRNA may result in either enhanced or reduced MDR-1 expression, however. Boyerinas *et al.* [82] found that insulin-like growth factor 2 mRNA binding protein 1 (IGF2BP1, also known as IMP-1) is repressed by the Let-7 group of miRNAs. Because IMP-1 is an RNA binding protein that stabilizes other mRNA transcripts, including *MDR-1*, Let-7 miRNA-dependent destabilization of *IMP-1* mRNA results in diminished MDR-1 expression. In fact, the reduced Let-7 miRNA levels observed in ovarian cancer are associated with MDR-1 upregulation and taxane resistance. In addition, upregulation of miR-130a and miR-296 are both associated with enhanced MDR-1 expression and increased drug resistance, although the primary targets responsible for these miRNA-dependent effects on MDR-1 levels are as yet unclear [83,84].

Drug resistance due to the breast cancer resistance protein (ABCG2/BCRP), another ABC transporter involved in cancer drug resistance, is also regulated by miRNAs. In MCF7 breast cancer cells and in K562 CML cells, miR-212 targets the *ABCG2* 3′-UTR, resulting in diminished ABCG2 expression and decreased resistance to mitoxantrone or imatinib, respectively [85,86]. In a similar manner, Dong *et al.* [87] found that miR-31 targets the 3′-UTR of the *ABCB9* mRNA transcript, causing its downregulation and decreased resistance to cisplatin in non-small-cell lung cancer (NSCLC) cells. Song *et al.* [88] showed that miR-495 targets the copper transporting P-type adenosine triphosphatase (*ATP7A*) 3′-UTR in NSCLC, causing its level to diminish in parallel with decreased resistance to cisplatin. Overall, the results of these studies indicate that drug resistance in cancer cells caused by enhanced expression of key ABC transporters, such as MDR-1, is often associated with decreased expression of miRNA species that destabilize their mRNA transcripts. Investigators are beginning to explore nanoparticles and other pharmaceutical strategies for the co-delivery of chemotherapeutic agents combined with miRNA species designed to overcome drug resistance by targeting MDR-1 or other efflux pumps [for review, see 69].

MicroRNAs contribute to cancer drug resistance via altered drug metabolism

Resistance to cancer chemotherapeutic agents may also develop via altered drug metabolism. Zong *et al.* [89] found that increased miR-130b expression in A2780 human ovarian cancer cells leads to upregulation of both glutathione *S*-transferase π (GST-π) and MDR-1, which were associated with increased resistance to paclitaxel. In LS174T colorectal cancer cells, Raynal *et al.* [90] showed that enhanced chemoresistance to SN38, the active metabolite of irinotecan, occurred through increased expression of the UDP-glucuronos-

Table 2. Cancer drug resistance involving MDR-1/ABCB1 influenced by miRNAs.

Drug-resistant human tumor cell model	miRNA	Drugs	Molecular targets	Ref.
Ovarian cancer cell lines	miR-130a	Cisplatin	MDR-1	[83]
Ovarian cancer cell lines	miR-27a, miR-451	Vinblastine, doxorubicin	MDR-1	[237]
Ovarian cancer cell lines	miR-130b	Cisplatin, paclitaxel	MDR-1, GST-π	[89]
Breast cancer cells	miR-451	Doxorubicin	MDR-1	[79]
Gastric cancer cells	miR-27a	Cisplatin, doxorubicin, vincristine, 5-fluorouracil	MDR-1	[238]
Esophageal cancer cells	miR-296	Doxorubicin	MDR-1	[84]
Breast cancer cells	miR-298	Doxorubicin	MDR-1	[78]
Embryonic kidney cells	miR-451	Doxorubicin	MDR-1	[79]

yltransferases *UGT1A1*, *UGT1A9* and *UGT1A10* via a pregnane X receptor (PXR)-dependent mechanism and the *PXR* 3′-UTR is targeted by miR-148a, leading to diminished PXR levels [91].

MicroRNAs contribute to cancer drug resistance via overexpression of target molecules

Overexpression of the molecular target for a cancer drug represents another mechanism for drug resistance. Taxanes, such as paclitaxel, bind to the β subunit of tubulin and induce growth arrest in the G2/M phase of the cell cycle by stabilizing microtubules found in mitotic spindles. However, increased levels of various β-tubulin isoforms by tumor cells leads to taxane resistance. Lobert *et al.* [92] found that introduction of miR-100, whose levels are lower in MCF7 breast cancer cells after being exposed to paclitaxel, also led to reduced mRNA levels for the β-tubulin 2A and β-tubulin 3 isoforms, decreased β-tubulin 3 protein levels and diminished resistance to paclitaxel.

MicroRNAs contribute to cancer drug resistance via enhanced survival anti-apoptosis pathways

Drug resistance may also occur via the enhancement of antiapoptosis/cell survival pathways (Table 4, Figure 3). Xia *et al.* [93] found that vincristine-resistant SGC7901/VCR human gastric adenocarcinoma cells exhibited reduced levels of miR-15b and miR-16, which target the 3′-UTR of *BCL2* mRNA transcripts. Re-introduction of miR-15b or miR-16 into SGC7901/VCR cells caused BCL2 expression to decrease along with diminished resistance to vincristine. As an example of indirect regulation by miRNA of a cell survival pathway, Zimmerman *et al.* [94] observed reduced expression of miR-181 in imatinib-resistant MYL-R CML cells along with increased expression of *LYN*, an Src family kinase associated with resistance to imatinib. Inhibition of LYN caused miR-181 levels to increase, which led to decreased expression of the BCL2 family member MCL2, whose 3′-UTR is targeted by miR-1841. Increased expression of the neutral apoptosis inhibitory protein (NAIP) was observed in cisplatin-resistant SK-N-AsCis24 neuroblastoma cells along with decreased expression of miR-520f, which was found to target the 3′-UTR of *NAIP* mRNA transcripts. Transfection with miR-520f caused reduced expression of NAIP and decreased resistance to cisplatin and etoposide. The phosphatase and tensin homolog (PTEN) tumor suppressor is also regulated by miRNA species. Yang *et al.* [95] showed that *PTEN* expression is often reduced in ovarian cancer, miR-214 is often upregu-

Table 3. Cancer drug resistance involving ABC transporters influenced by miRNAs.

Drug-resistant human tumor cell model	MicroRNA	Drugs	Molecular targets	Ref.
Chronic myelogenous leukemia cells	miR-212, miR-328	Imatinib	ABCG2	[85]
Non-small-cell lung cancer cells	miR-31	Cisplatin	ABCB9	[87]
Non-small-cell lung cancer cells	miR-495	Cisplatin	ATP7A	[88]
Gastric cancer cells	miR-129–5p	Cisplatin, vincristine, 5-fluorouracil	ABCB1, ABCC5, ABCG1	[81]
MCF7 breast cancer cells	miR-100	Taxanes	β-tubulin	[92]

lated, that the 3′-UTR of *PTEN* mRNA transcripts is targeted by miR-214, and that decreased resistance to cisplatin results when miR-214 antagomirs are introduced. Chen *et al.* [96] found that the cisplatin-resistant neuroblastoma cell lines CisR SH-SY5Y and CisR BE(2)-M17, which bears an *MYCN* gene amplification, expressed increased levels of miR-21 and decreased levels of PTEN compared with their parental cell lines. Introduction of miR-21 into the parental cell lines caused *PTEN* to decrease and resistance to cisplatin to increase. Garofalo *et al.* [97] showed that miR-221 and miR-222, which are upregulated in NSCLC cells and hepatocellular carcinomas, target a site in the *PTEN* 3′-UTR that causes reduced PTEN expression and increased resistance to the TNF-related apoptosis inducing ligand (TRAIL). Similarly, miR-93, which is upregulated in cisplatin-resistant ovarian cancer cells, also targets the 3′-UTR of *PTEN* to reduce its expression and provide enhanced drug resistance [98]. The 3′-UTR of tumor suppressor protein p27KIP1 is also targeted by miR-221 and miR-222 [99], and upregulation of these miRNAs in breast cancer cells [100] and in NSCLC cells [101] is associated with decreased expression of P27KIP1 and increased resistance to tamoxifen or TRAIL, respectively.

The results of these and other investigations of mechanisms of drug resistance establish important functional roles for miRNA. Changes in miRNA levels may be particularly important during early tumor development in providing growth and survival conditions that are more suitable for further adaptive changes to occur. Further studies are needed to evaluate the relative significance of these changes in miRNA in future cancer therapy decision making.

MiRNA in assessment of drug efficacy

Many studies have shown that a large number of miRNAs are associated with diseases and the mechanisms of miRNA involved in pathogenesis are very complicated [10–14]. The Human microRNA Disease Database (http://cmbi.bjmu.edu.cn/hmdd) listed 572 miRNA genes associated with 378 diseases from 3511 articles. On the other hand, an increased number of studies demonstrated that miRNAs could be used to assess drug efficacy and toxicity [102].

Circulating miRNAs as a noninvasive determinant of response to drugs

A major interest in current pharmacology is in the utilization of miRNAs as predictors of drug efficacy, which could have a tremendous impact on the optimization of drug treatment. As miRNA expression has been reported to be affected by drugs and since miRNAs themselves may affect drug metabolism and toxicity, miRNA expression can be also utilized as potential biomarkers of drug efficacy.

Of particular interest as potential biomarkers are the recently explored circulating miRNAs, a new class of cellular messengers that exist in most biological fluids in a relatively stable form [103]. Potential advantages in using circulating miRNAs as biomarkers include: their remarkable stability in body fluids such as plasma, serum, urine, cerebrospinal fluid, semen, saliva and even breast milk; miRNAs can be detected and quantified using simple methods such as PCR; and the fact that miRNAs can often be obtained from patients easily using noninvasive or minimally invasive procedures [103–108]. Circulatory miRNAs remain stable even under conditions that would otherwise degrade other types of RNA (i.e., mRNA), such as dramatic changes in pH and room temperature storage conditions [109]. The stability of circulating miRNAs may be attributed to the fact that they can be secreted from the donor cells packaged within exosomes or microvesicular components, which provide some protection from miRNA degradation and that exosomal miRNA transported by the circulatory system can be taken up by recipient cells in a manner that affects their behavior as well [110–112].

MiR-21 & miR-200bc family as universal predictors of response to cancer therapy

It is anticipated that circulatory miRNAs may serve as useful predictive biomarkers of response to chemotherapy. Significant alterations in circulating miRNA expression have been reported in cancer patients in comparison with healthy individuals [109,113]. Furthermore, a number of *in vitro* investigations describe altered patterns of miRNA expression in cells resistant to anticancer drugs [114,115].

MiR-21 is one of the most studied onco-miRNAs, whose overexpression has been detected in virtually all human cancers [116]. It negatively regulates the expression of the tumor-suppressor gene *PTEN* as well as the proapoptotic protein *BAX*. Thus, overexpression of miR-21 is associated with increased proliferation and tumor invasiveness paralleled by the inhibition of apoptosis. In turn, silencing of miR-21 leads to decreased cellular proliferation, reduced invasiveness and diminished tumor metastatic potential along with the induction of cell cycle arrest.

Accumulating evidence clearly indicates that higher expression of miR-21 is associated with resistance to a number of chemotherapeutic drugs. For instance, high expression of miR-21 was associated with cisplatin resistance in lung cancer patients [117]. Interestingly, when the cisplatin-resistant A549 lung adenocarcinoma cells were transfected with anti-miR-21, it led to upregula-

Table 4. Cancer drug resistance involving survival pathways influenced by miRNAs.

Drug-resistant human tumor cell model	MicroRNA	Drugs	Molecular targets	Ref.
Gastric cancer cell lines	miR-15b, miR-16	Vincristine	Bcl-2	[93]
Chronic myelogenous leukemia cell lines	miR-181b, miR-181d	Imatinib	LYN, Mcl-1	[94]
Neuroblastoma cells	miR-520f	Cisplatin	NAIP	[239]
Neuroblastoma cells	miR-21	Cisplatin	PTEN	[96]
Ovarian surface epithelial cells	miR-214	Cisplatin	PTEN	[95]
Ovarian cancer cell lines	miR-93	Cisplatin	PTEN	[98]
Hepatocellular carcinoma cell lines	miR-221, miR222	TRAIL	PTEN	[97]
Ovarian cancer cell lines	miR-93	Cisplatin	PTEN	[98]
Breast cancer and non-small-cell lung cancer cells	miR-221, mir-222	Tamoxifen, TRAIL	P27KIP1	[100,101]

tion of *PTEN*, downregulation of antiapoptotic protein *BCL2*, and overall modulation of response to cisplatin. Aberrant expression of miR-21 has been also associated with resistance to 5-fluorouracil, docetaxel, gemcitabine and temozolomide in pancreatic and other human cancers [118,119].

MiR-200b is a member of the miR-200-family, transcribed from the miR-200b/a/429 cluster on chromosome 1. This important miRNA has tumor-suppressor characteristics evidenced by inhibition of epithelial-to-mesenchymal transition (EMT) and angiogenesis [120]. MiR-200b targets a number of angiogenic growth factors, including the *PDGF*, *VEGF* as well as the pro-angiogenic factors cytokine *CXCL1* and *IL*-8 [121]. Alterations in miR-200b expression play a critical role in EMT, and its loss is associated with increased metastatic potential.

MiR-200b, aside from being found downregulated in cancerous tissue, has been also tightly associated with resistance to numerous chemotherapeutic drugs. In contrast to miR-21, the expression of miR-200b was reported to be significantly lower in A549 cells resistant to cisplatin [122]. Induction of miR-200b expression, together with miR-429, has led to modulation of the cellular response to cisplatin via downregulation of two antiapoptotic proteins, *BCL2* and *XIAP* [122]. Similarly, downregulation of miR-200b has been associated with resistance to docetaxel [123], and restoration of miR-200b expression has resulted in restoration of cellular sensitivity to docetaxel [124]. Altogether, these studies suggest that the expression status of miR-21 and miR-200b may potentially serve as predictive surrogate biomarkers for patients' responses to chemotherapy.

MiRNAs in prediction of response to sunitinib

Sunitinib is a tyrosine kinase angiogenesis inhibitor that works via targeting the *VEGFR*, *PDGFR* and c-Kit. In humans, sunitinib is now used to treat metastatic renal cell carcinoma and colon cancers and currently sunitinib is in clinical trials for glioblastoma. A set of interesting studies has been performed on the prediction of response to sunitinib treatment in experimental systems and cancer patients.

In a murine model of pancreatic cancer (tumor-bearing RT2 mice), a 7-day treatment with sunitinib led to normalization of expression of miR-424, miR-126 and miR-21, all of which were found upregulated in angiogenic islets prior to sunitinib treatment [125]. Furthermore, the authors showed positive dynamics in the downregulation of other miRNAs, including miR-451, miR-199*, miR-223, miR-143, miR-145, miR-10b and miR-126*. A recent study using Hs766T human pancreatic carcinoma cells has shown that silencing of miR-21 (one of the miRNAs upregulated in the mouse model) in combination with low amounts of sunitinib resulted in a strong antitumor effect [126]. Similar results were observed in another study, where the oligonucleotide-mediated silencing of miR-21 in U87 human malignant glioblastoma cells enhanced the antitumor effect of sunitinib [127].

Berkers *et al.* [128] performed an analysis of miRNA expression in freshly frozen clear cell renal carcinoma tissue samples from patients who received sunitinib as a first-line targeted therapy. This study involved only patients with synchronous metastasis. A significant decrease in the expression of miR-141 was noted in patients with progressive disease in comparison with patients with at least 1 year of progression-free disease. The role of miR-141 in progression of clear cell renal carcinoma was confirmed *in vitro*, where its re-introduction was associated with reversal of epithelial-to-mesenchymal transition and increased sensitivity to a hypoxic environment. A subset of other miRNAs, differentially regulated between the patients who

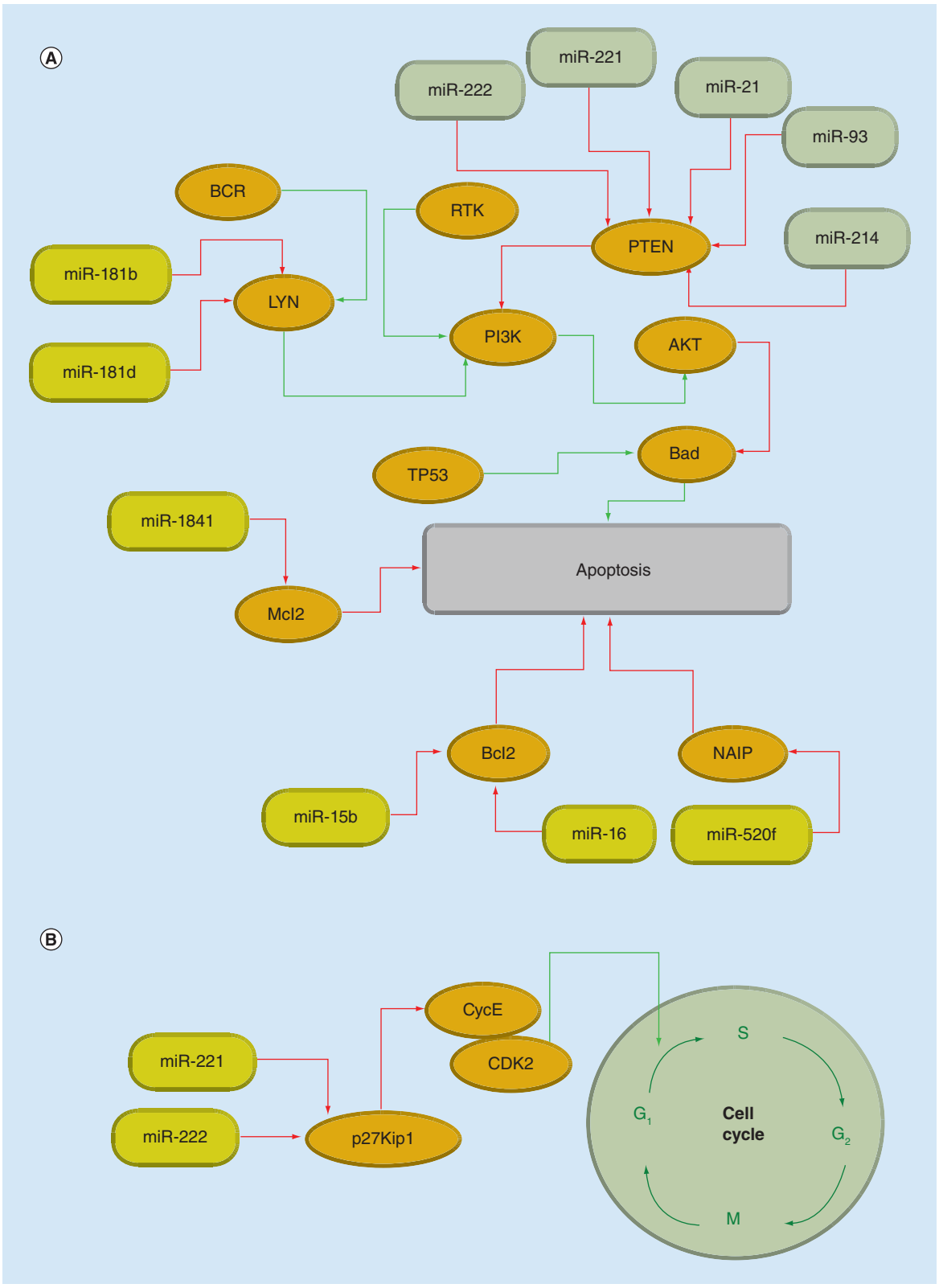

Figure 3. Cancer drug resistance involving apoptosis (A) and survival (B) pathways influenced by miRNAs.
Protein factors are depicted in orange ovals. MicroRNAs that are upregulated in drug-resistant cancer cells are presented in green ovals. MiRNAs which are present in lower levels are given in yellow boxes. Interactions leading to suppressed expression or reduced concentrations are marked with red arrows and interactions leading to stimulated activity or higher concentrations are marked with green arrows.

responded to sunitinib treatment versus the nonresponders, included miR-520g, miR-155, miR-526b (all upregulated) and miR-144 and miR-376b (both downregulated).

Another study conducted by Prior *et al.* [129], performed at the University of Navarra Clinic (Spain), reported miR-942 as the most accurate predictor of sunitinib efficacy in a cohort of patients with metastatic renal cell carcinoma. Furthermore, increased expression of miR-942 was also significantly associated with decreased time to progression and overall survival.

Of particular interest is a study on miRNA expression in the peripheral blood of patients with advanced renal cell carcinoma who received sunitinib as a first-line therapy [130]. Overall, the authors identified 28 miRNAs related to response to sunitinib. Among them, 12 predictive signatures of poor response were identified, as well as 12 predictive signatures of prolonged response. Interestingly, none of the miRNAs found to be differentially expressed in this study was reported in the previous studies, except for miR-126*, which was found to be downregulated in response to successful sunitinib treatment in the mouse model of pancreatic cancer [125]. Also, in the study of 380 circulating miRNAs in patients receiving sunitinib therapy for metastatic colon cancer, miR-296 was associated with a statistically significant correlation in the patient's clinical outcome [131]. Specifically, a decrease in the levels of circulating miR-296 levels correlated with shorter survival and poor response to sunitinib therapy.

MiRNAs as predictors of responses to other drugs

Recent studies indicate that miRNAs may serve as predictive biomarkers for treatments with other drugs in addition to those used in cancer therapy. For instance, Fujioka *et al.* [132] reported that the levels of miRNAs let-7d and let-7e in Crohn's disease patients were significantly increased in the group of patients who achieved clinical remission by treatment with the drug infliximab. Furthermore, several miRNAs were proposed as predictive tools of glucocorticoid response in patients with inflammatory bowel diseases [133]. Specifically, an association was demonstrated that several SNPs may alter the function or expression of miR-125, miR-181, miR-499 and miR-519 in patients with nonresponsiveness to glucocorticoid treatment [134].

MiRNAs in assessment of drug safety

Taking into account a growing number of studies that indicate miRNA involvement in drug-induced toxicity in different organs, such as liver, heart, kidney, muscle, central nerve system and reproductive organs [102], it has been suggested that miRNAs can be utilized in the assessment of drug safety. Drug-induced liver injury and cardiotoxicity are among the primary concerns in the process of drug development, although renal toxicity is also an important issue.

Assessment of hepatotoxicity

Drug-induced liver injury (DILI) is a leading cause of acute liver failure. DILI is also a major reason for drug failure at the development stage and a cause for the withdrawal of approved drugs from the market [135]. Measuring the levels of commonly used biomarkers, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) and bilirubin in blood samples has been a standard clinical practice for detecting liver injury in patients for many years [136]. However, these biomarkers lack in sensitivity and specificity for early liver injury diagnosis and for obtaining accurate prognoses for liver disease. For instance, nonhepatic injuries may also affect blood levels of ALT and AST. Elevated ALT and AST levels have been associated with other types of physiological events, such as heart, kidney or skeletal muscle injury, intramuscular drug injections and intense physical exercise [137]. In addition, blood levels of ALT and AST do not always correlate well with histomorphological changes associated with liver damage [136]. More reliable biomarkers are needed for the early detection of liver injury and, it is hoped, for facilitating individualized patient treatment strategies. In recent years, novel hepatic biomarkers have been emerging from preclinical studies and clinical studies. HMGB1 (highly mobility group box-1; marker of necrosis), K18 (keratin-18; marker of necrosis and apoptosis) and GLDH (glutamate dehydrogenase, marker of mitochondria damage) have been identified as alternative biomarkers of liver toxicity with improved sensitivity [138]. Additional protein biomarkers including PNP (purine nucleoside phosphorylase), SDH (sorbitol dehydrogenase), ARG-1 (arginase 1) and Egr1 (early growth response protein1) have been also reported to be associated with hepatotoxicity [139,140].

Circulating miRNAs have received particular attention as potential biomarkers of liver injury recently because of the advantages they can provide in terms of greater stability in the types of patient specimens that are encountered most commonly (blood and urine) and the simple and cost-effective laboratory methods typically used for miRNA analysis. During organ damage, miRNAs are often released into biofluids. Thus, miRNAs in the blood or urine are being studied as noninvasive biomarker candidates for detecting hepatic diseases and toxicity [141,142]. Furthermore, useful liver-specific miRNAs indicative of liver injury

have been identified. For instance, miR-122 is predominantly expressed in liver and its level of expression is 5000- to 100,000-times higher than in other tissues, such as the brain, heart, kidney, lung, ovary, spleen, testes and thymus [142].

Various studies have been conducted to investigate changes in miRNAs that occur in response to challenges by known hepatic toxicants, such as acetaminophen, benzo [*a*] pyrene, carbon tetrachloride, dioxin, tamoxifen and herbal dietary supplements [138,143–148]. Most studies have focused on acetaminophen (*N*-acetyl-p-aminophenol; APAP) because acetaminophen poisoning is a common cause of DILI in humans, acetaminophen is one of the most extensively studied liver damaging agents in rodents and humans, and a great deal is known about the hepatotoxic effects of acetaminophen.

A study by Ward *et al.* [149] investigated the use of circulating miRNAs as biomarkers for diagnosing acetaminophen poisoning and changes in circulating miRNAs during treatment of N-acetyl cysteine (NAC), an antidote for acetaminophen poisoning. These authors reported that a set of 11 circulating miRNAs could be used in patients undergoing NAC treatment to distinguish acetaminophen hepatotoxicity from ischemic hepatitis (miR-122–5p, miR-27–3p, miR-21–5p, miR-194–5p, miR-193a-5p, miR-125b-5p, miR-1290, miR-4524a-3p, miR-23b-5p, miR-28–3p and miR-1247). In addition, the authors found that elevated circulating miRNAs decreased to normal levels during NAC treatment in most patients (44 out of 49 patients).

MiRNAs have been extensively investigated in DILI using serum/plasma obtained from animal models and from patients, primarily those with APAP overdoses. The results of these studies, aside from the knowledge that DILI is associated with altered miRNA patterns, have provided several important insights.

First, these studies provided convincing evidence that miRNAs may not only serve as surrogate biomarkers of DILI but can be utilized in assessment of drug safety. For instance, a comprehensive study that investigated the expression of plasma miRNAs in mouse models of acute and chronic liver injury reported that miRNA hierarchical clustering analysis clearly discriminated between these two types of liver injury [150]. Furthermore, the miRNA expression profiles were different in response to the administration of different liver damaging agents, including APAP, α-naphthyl isothiocyanate and CCl_4. The authors also showed that these miRNAs were not affected in their experimental model of cholestasis, which was caused by bile duct ligation.

Second, parallel analyses of data obtained from the rodent models and data obtained from patients with acute liver injury revealed many commonalities in their miRNAs responses. For instance, elevated levels of circulating miR-122 and miR-102 were detected in both animal models and in the plasma of APAP-overdosed patients [138,145,150,151]. These consistent results suggest that miR-122 and miR-102 may potentially be used as predictors of acute liver injury in preclinical drug safety assessment, thus decreasing the probability of negative health effects in humans and leading to an overall decrease in the price of drug development.

Third, a report by Ward *et al.* [152] suggests that a specific set of miRNA signatures may be associated with lethal doses of drugs. For instance, miR-575–5p, miR-135a* and miR-466g were greatly upregulated (over 100-fold) while miR-342–3p, miR-195 and miR-375 were greatly downregulated (over 100-fold) in mice exposed to the lethal doses of APAP (500 mg/kg) in comparison with those exposed to a sublethal dose (150 mg/kg).

Further application of high-throughput sequencing allowed the identification of specific circulating miRNA signatures associated with APAP overdose [145]. Importantly, the data in this study obtained from human subjects also confirmed the results of previous findings in animal models, where the overexpression of miR-107, miR-122, miR-130a, miR-148a, miR-192, miR-22, miR-27b and miR-30a was also identified in the plasma of APAP-overdosed mice [153]. Furthermore, the authors confirmed very low baseline levels with little variability in healthy individuals and sharp increases in the serum of APAP-overdosed patients of two miRNAs: miR-122 and miR-192 [145]. Similar results were reported by Starkey Lewis and colleagues [141], where they observed the expression of miR-122 and miR-192 to be more sensitive and more specific than ALT in evaluating liver injury.

Another study by Antoine *et al.* [138] reported elevated levels of miR-122 detected in the plasma of APAP overdosed patients soon after the initial poisoning occurred, including patients with ALT within the normal range. This finding also correlated with animal studies that report elevated levels of miR-122 in the plasma of mice overdosed with APAP, even before the increase in the levels of plasma aminotransferases, and this also correlated with the extent of hepatocellular injury [150]. Altogether, these findings suggest that the expression of circulatory miR-122 may potentially serve as a robust and reliable biomarker of hepatotoxicity in preclinical assessment of drug safety, as well as acute liver injury in patients.

MiRNAs in assessment of cardiotoxicity

Cardiotoxicity is one of the major obstacles in the process of drug development and accounts for about

9% of withdrawals of prescription drugs [154]. Furthermore, a number of anticancer drugs, such as 5-fluorouracil, anthracyclines, imatinib and trastuzumab, may induce cardiotoxicity [155]. Effective safety screening to identify drug-induced cardiotoxicity is a serious issue in drug development. In drug industry, nonclinical models are widely used to predict cardiac toxicity [156], for example, the utilization of telemetry to record electrocardiograpm and heart rate in rodents [157] and the application of cost-effective hERG assays to predict QT prolongation [158]. However, the potential for a new drug to cause cardiotoxicity may not be detected until after clinical trials in humans have begun. In some cases, drug-associated cardiotoxicity may not be observed until after drug approval [159,160]. The potential adverse effects of cardiotoxicity are arrhythmias, hypertension, abnormal left ventricular ejection fraction (LVEF) and eventually heart failure [161]. Several methods have been applied to monitor the cardiotoxic effects of drugs. The commonly used approaches by clinicians are Doppler echocardiography, echocardiographic LVEF and radionuclide angiography [161]. The limitation of these techniques is that they are relatively insensitive. The most sensitive test is invasive endomyocardial biopsy; however, this highly invasive procedure may cause severe life-threatening consequences [162]. The most widely used serum protein biomarkers for cardiotoxicity are cardiac troponin I (cTnI) and cardiac troponin T (cTnT) [163]. Cardiac troponins, however, exhibit rapid clearance from the circulation and are elevated only during a 3–12 h period after tissue injury has occurred [161,164,165]. Other biomarkers, such as cardiac myoglobin and creatine kinase isoenzymes, have reduced sensitivity and are not suitable for rapid determination of cardiotoxicity [154]. Thus, there is an urgent need for early and sensitive biomarkers for the diagnosis of drug-induced cardiac tissue injury.

Over the past few years, the study of miRNAs as early biomarkers of drug-induced cardiotoxicity has become a burgeoning research topic. A subset of cardiac-specific miRNAs has been determined, including miR-1, miR-133a, miR-133b, miR-206, miR-208a and miR-499 [151,166,167]. These miRNAs have been detected in the blood in studies of experimental acute cardiotoxicity using rodents and in human myocardial injury. Utilization of these miRNAs as relatively noninvasive circulatory predictors of cardiotoxicity may have a great potential on drug safety assessment, especially in preclinical models.

An *in vivo* mouse study conducted by Desai *et al.* [168] found that the expression of 24 miRNAs was significantly altered in mouse hearts treated with cumulative doses of doxorubicin. Among the differentially expressed miRNAs, miR-34a was highly and specifically expressed and it showed a significant dose-related upregulation at all cumulative doxorubicin doses. Moreover, changes in miR-34a expression occurred before the observation of cardiac damage detected by release of cardiac cTnT in plasma or cardiac pathology. In addition, miR-208 increased with a similar time course with the plasma cTnI level. Another study by Liu *et al.* [160] compared the role of miR-208a in isoprenaline-treated wild-type C57BL/6J mice and C57BL/6J mice that were heterozygous for Sod2 ($Sod2^{+/-}$). They concluded that increases in miR-208a detected in plasma correlated more closely with isoprenaline-induced myocardial damage than cTnI levels in plasma in both types of mice.

Of particular interest is miR-208, which is expressed exclusively in the heart, while the other reported miRNAs can be also detected in skeletal muscles [169]. miR-208 has been reported in the plasma of rats with isoproterenol-induced myocardial injury and, importantly, showed a similar time-course with plasma cTnI levels [166]. Subsequent studies have shown that plasma levels of miR-208 were consistently increased through 24 h following the administration of isoproterenol in rats, as well as after repeat-dose treatment [167].

Horie *et al.* [170] reported a significant upregulation of miR-146a in neonatal rat cardiac myocytes after treatment with doxorubicin, a drug with known cardiotoxicity [171], and that *ErbB4* represents a target gene whose expression is suppressed by miR-146a. The authors also showed that either overexpression of miR-146a or *ErbB4* knockdown increased doxorubicin-induced apoptotic cell death. Their results suggest that inhibition of miR-146a may provide a potential treatment strategy for doxorubicin-induced heart injury. Administration of doxorubicin for 2 and 4 weeks also induced the expression of miR-208, as well as miR-215, miR-216b, miR-34c and miR-367 in rat heart, with the effects being more pronounced after 4 weeks [172]. Unfortunately, these authors did not perform an analysis of circulatory miRNAs. Nishimura *et al.* [167] reported that short-term treatment with doxorubicin was associated with elevated levels of plasma miR-206 (at 8 and 24 h after dosing) and miR-1, miR-133a and miR-133b (at 24 h after dosing only). The plasma levels of miR-208 remained unchanged in these animals, in contrast to animals treated with isoproterenol (which is capable of causing acute myocardial injury), indicating that circulatory miR-208 may serve as a diagnostic surrogate biomarker of cardiotoxicity, but not a predictive one. It is also plausible to hypothesize that miR-216 could serve as a predictive biomarker of cardiotoxicity, because alterations in miR-216 expression can be detected in the heart earlier than histopathologically observed tissue lesions [172], however further experiments are needed to confirm this hypothesis.

MiRNA in drug-induced kidney toxicity

Total urinary protein, glucose and N-acetyl-β-(D)-glucosaminidase and serum creatinine and urea nitrogen (BUN) have been used as biomarkers for nephrotoxicity. Similar to the liver injury biomarkers ALT and AST, elevation of these biomarkers of nephrotoxicity generally reflect well-established renal damage and does not provide adequate sensitivity for the detection of early events associated with kidney injury.

Efforts have been made to uncover sensitive and predictive biomarkers of nephrotoxicity using various technologies. A panel of protein biomarkers, showing higher sensitivity and specificity than the conventional biomarkers, such as blood creatinine and BUN, has been identified and characterized. Other protein biomarkers, such as kindney molecule-1, albumin, a-GST, a1-microglobuin, clusterin, heart-typefatty acid-binding protein, netrin-1 and neutrophil gelatinase-associated lipocalin have been used to monitor kidney toxicity [139,173]. The use of these biomarkers in rodents for evaluating kidney injury has been approved by the FDA and European Medicines Agency (EMA) [174]. Greater understanding of the nature of circulating miRNAs has sparked interest in their use as additional biomarkers in drug-induced nephrotoxicity.

The use of miRNA for the detection of nephrotoxicity by cisplatin, a well-known nephrotoxic compound, was first reported by Blatt *et al.* [175]. The induction of miR-34a was observed in cultured proximal tubular cells and in renal tissues of mice treated with cisplatin. The induction of miR-34a was associated with p53 activation and also with the outcome of cisplatin-induced toxicity. Kanki *et al.* [176] investigated changes in urinary miRNA in rats dosed with cisplatin. The expression of a set of 25 miRNAs was increased in urine samples, whereas all were decreased in kidney tissues. The authors suggested that the appearance of miRNAs in urine may be due to leakage of miRNA from the injured kidney cells. Moreover, the change of these urinary miRNA correlated with the severity of necrosis in proximal tubules. Out of 25 miRNAs altered, four miRNAs (let-7g-5p, miR-93–5p, miR-191a-5p and miR-192–5p) seemed to have similar sensitivity compared with the serum biomarkers BUN and creatinine, indicating these urinary miRNA can be potentially used as noninvasive biomarkers for detection of cisplatin-induced kidney toxicity [176]. A rat study conducted by Saikumar *et al.* [177] examined changes in miR-21, miR-155 and miR-18a associated with kidney injury induced by ischemia/reperfusion and gentamicin treatment. Their study revealed that all three miRNAs were upregulated in kidneys, whereas opposite changes (downregulation) were observed for miR-21 and miR-155 in both blood and urine samples. The increase of miR-21 and miR-155 corresponded to the extent of histological damage, implying that miR-21 and miR-155 may play a role in the pathogenesis of kidney injury and may serve as potential biomarkers of nephrotoxicity.

Future perspective

Since the discovery of miRNAs in 1993 [178] and the recognition of their regulatory function [179], over 38,000 articles and reviews have been published describing these molecules diverse functions. Initially on the roles of miRNA in development [180,181], disease [182,183] and cancer [184,185] were recognized, as well as the tissue specificity of certain miRNAs [186]. Not long after these discoveries, miRNAs were found to be regulated under conditions of toxicity [147,187]. The extent of information generated is such that over a dozen databases exist to provide analysis of miRNA experimental data and insight into variants, miRNA targets and regulatory networks [188–197]. With the application of next-generation sequencing technology (RNA-seq) to miRNA analysis [198–203], the discovery of novel small RNAs [204] and their role in disease [205] and toxicology will be continuing to expand.

Thus, the enormous potential for diagnosing and/or treating pathological conditions has clearly presented itself. With the discovery of miRNA in serum and plasma [206–208], the possibilities for diagnosis have truly blossomed. In spite of this promise, circulating miRNA still has yet to come into clinical and preclinical use [209]. While Rosetta Genomics has described the development of a commercial miRNA-based test that differentiates between the four main types of lung cancer (squamous cell carcinoma, nonsquamous non-small-cell lung cancer, carcinoid and small cell carcinoma) [210], the miRNA-based assay has yet to be approved as an IVD (*in vitro* diagnostic) by the FDA. We expect there will be more endeavors in the future to surmount hurdles faced in pursuing 'biomarker qualification' [211] and the difficult process in taking a molecular diagnostic from a research discovery to a regulatory-approved clinical diagnostic [212].

Nonetheless, the rapid growth of the field suggests that miRNAs will indeed play an important role in guiding cancer treatment, drug development and ultimately disease treatment. However, the single battery of microRNA data is limited to help fully unreveal the complicity of a biological system. Therefore, comprehensive integration and interpretation of multiple types of '-omic' data, such as genomic, epigenomic, trascriptomic, proteomic and metabolomic data, will be an important approach to interrogate the mechanisms of biological functions, healthy and diseased states in a systems biology approch [213,214]. The top priority of

such studies will aim at a better understanding the role of miRNAs in various pathological conditions, individual phenotypes and drug or chemical responses. With the efforts being made, we are looking forward to such a day in the near future when the impact of miRNAs on cellular functions and phenotypic traits is fully realized and understood.

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Disclaimer

The views presented in this article do not necessarily reflect those of the US FDA.

Executive summary

Biological facts of miRNA

- MiRNAs are a family of small (~22nt) noncoding RNAs widely expressed in eukaryotic cells.
- MiRNAs can regulate gene expression post-transcriptionally by promoting mRNA degradation or inhibition of mRNA translation.
- A miRNA can target many mRNA transcripts and an mRNA transcript can be target by many miRNAs.
- MiRNAs are involved in a great variety of biological events.

Impact of miRNA on pharmacogenomics: interindividual variability of DMETs by miRNA modulation

- The expression of human drug metabolizing enzymes and transporters (DMETs) exhibits huge interindividual variability, which contributes to interindividual variability in drug efficacy and safety.
- MiRNA expression is affected by genetic variants, environment stimuli/stressors and drug–drug interactions.
- MiRNAs manifest characteristics of interindividual variability in the expression of DMETs.

Roles of miRNA species in mediating intrinsic & acquired resistance to cancer therapeutics

- Heritable single nucleotide polymorphisms affecting miRNA function can affect intrinsic drug resistance.
- Exosomal transfer of miRNA and other products from drug-resistant tumor cells may contribute to drug resistance in recipient cells.
- MiRNA may contribute to drug resistance via enhanced drug efflux, altered drug metabolism, induced overexpression of drug target molecules, or upregulated cell survival pathways.

MiRNA in assessment of drug efficacy

- Circulating miRNAs, a new class of cellular messengers that exist in biological fluids are the most promising biomarkers in the assessment of drug efficacy and toxicity.
- Data obtained from the rodent models and from humans reveal many commonalities in their miRNA responses.
- A specific set of miRNA signature is associated with a specific drug.

MiRNAs in assessment of drug safety

Assessment of hepatotoxicity

- Elevated levels of miR-122 have been detected in the plasma of high-dose acetaminophen treated animals and acetaminophen overdosed patients, thus miR-122 can be used as a predictor of acetaminophen toxicity.
- Circulating miRNAs can serve as biomarkers for detecting drug-induced liver injury.

MiRNAs in assessment of cardiotoxicity

- Utilization of miRNAs as surrogate circulatory biomarkers of cardiotoxicity holds a great potential on drug safety assessment.

MiRNA in drug-induced kidney toxicity

- In addition to newly identified and characterized protein biomarkers such as kidney molecule-1 (Kim-1), urinary miRNA may be useful biomarkers for detection of kidney injury.
- The increased level of miR-21 and miR-155 corresponded to the level of histological damage.

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